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I κ B β acts to both inhibit and activate gene expression at different stages of the inflammatory response

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Abstract

The activation of pro-inflammatory gene programs by nuclear factor- κ B (NF- κ B) is primarily regulated through cytoplasmic sequestration of NF- κ B by the inhibitor of κ B (I κ B) family of proteins¹. I κ B β , a major I κ B isoform, can sequester NF- κ B in the cytoplasm², although its biological role remains unclear. While cells lacking I κ B β have been reported^{3,4}, *in vivo* studies have been limited and suggested redundancy between I κ B α and I κ B β ⁵. Like I κ B α , I κ B β is also inducibly degraded, however upon stimulation by LPS, I κ B β is degraded slowly and resynthesized as a hypophosphorylated form that can be detected in the nucleus^{6–11}. The crystal structure of I κ B β bound to p65 suggested this complex might bind DNA¹². *In vitro*, hypophosphorylated I κ B β can bind DNA with p65 and cRel, and the DNA-bound NF- κ B:I κ B β complexes are resistant to I κ B α , suggesting hypophosphorylated, nuclear I κ B β may prolong the expression of certain genes^{9–11}. We now report that *in vivo* I κ B β serves to both inhibit and facilitate the inflammatory response. I κ B β degradation releases NF- κ B dimers which upregulate pro-inflammatory target genes such as tumor necrosis factor- α (TNF α). Surprisingly absence of I κ B β results in a dramatic reduction of TNF α in response to lipopolysaccharide (LPS) even though activation of NF- κ B is normal. The inhibition of TNF α mRNA expression correlates with the absence of nuclear,

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature

Author contributions PR characterized the mice and performed the majority of the experiments, MSH performed the immunoprecipitation experiments and helped in writing the paper, ML performed CIA experiments, DZ and APW performed generation of BMDM cells, AO performed some experiments, MLS and DB generated the knockout mice, CL and AH performed the RNAse protection assays, and SG conceived of the study and wrote the paper.

hypophosphorylated-I κ B β bound to p65:c-Rel heterodimers at a specific κ B site on the TNF α promoter. Therefore I κ B β acts through p65:c-Rel dimers to maintain prolonged expression of TNF α . As a result, I κ B $\beta^{-/-}$ mice are resistant to LPS-induced septic shock and collagen-induced arthritis. Blocking I κ B β might be a promising new strategy for selectively inhibiting the chronic phase of TNF α production during the inflammatory response.

To better understand the biological function of I κ B β we decided to study mice lacking the I κ B β gene. Homologous recombination was used to delete the majority of the I κ B β coding sequences (30–308 aa) including elements essential for binding to NF- κ B (Supplementary Fig. 2)^{6,12,13}. Absence of I κ B β was confirmed by immunoblotting of mouse embryonic fibroblasts (MEFs; Supplementary Fig. 2). Although I κ B β is expressed broadly including in hematopoietic organs (Supplementary Fig. 3a), the *I κ B β* knockout mice breed and develop normally without any obvious phenotypic defects.

NF- κ B and I κ B proteins function in an integrated network and hence reduced expression of one component may cause compensatory changes in levels of other proteins^{14,15}. However, expression levels of I κ B α , I κ B ϵ , p65, RelB, c-Rel, p105 and p100 were unaffected in *I κ B $\beta^{-/-}$* mice (Supplementary Fig. 3b). Increased NF- κ B activity has been observed in other I κ B knockouts^{16–18}, and increased basal NF- κ B reporter activity was observed in *I κ B $\beta^{-/-}$* MEFs (Fig. 1a). Electrophoretic mobility shift assays (EMSA) demonstrated increased basal NF- κ B activity in *I κ B $\beta^{-/-}$* cells (60%) (Supplementary Fig. 3c). Conversely, overexpression of I κ B β inhibits NF- κ B activation (Supplementary Fig. 3d). Thus I κ B β inhibits NF- κ B and degradation or loss of I κ B β contributes to NF- κ B activity. NF- κ B reporter assays reveal that absolute NF- κ B activity in response to LPS, IL-1 β or TNF α is slightly higher in the *I κ B $\beta^{-/-}$* than wild type (WT) cells (Fig. 1a). However, the kinetics of NF- κ B activation by EMSA, and the pattern of I κ B degradation by immunoblotting, in cells stimulated with LPS, IL-1 β or TNF α were not demonstrably different in *I κ B $\beta^{-/-}$* cells (Supplementary Fig. 4). Thus, loss of I κ B β results in a modest elevation in basal NF- κ B activity, while inducible NF- κ B activation is relatively unaffected.

NF- κ B regulates the expression of many genes, in particular those involved in inflammation and immune responses¹⁹. To determine whether I κ B β has a role in the inflammatory response, *I κ B $\beta^{-/-}$* and *I κ B $\beta^{-/+}$* mice were challenged with LPS. Surprisingly, *I κ B $\beta^{-/-}$* mice were significantly resistant to the induction of shock (Fig. 1b). We therefore examined the serum levels of the key acute phase cytokines TNF α , IL-1 β and IL-6²⁰ following LPS injection. In wild type mice TNF α production peaked 1 hour after LPS injection, while IL-6 and IL-1 β production peaked around 2 hours, in agreement with previous studies²¹. Although serum IL-6 and IL-1 β were reduced (~25%) in the *I κ B $\beta^{-/-}$* mice, the reduction of TNF α levels (>70%) was more striking (Fig. 1C). As the peak of serum TNF α precedes that of IL-1 β and IL-6, it is likely that the reduction of IL-1 β and IL-6 is secondary. As monocytes and macrophages are major sources for systemic TNF α , we analyzed LPS induced cytokines in thioglycollate-elicited peritoneal macrophages (TEPM). While equivalent macrophage populations were obtained from the mice (Supplementary Fig. 5a), TNF α , but not IL-6, production was drastically reduced in *I κ B $\beta^{-/-}$* TEPM (Fig. 1d).

To understand how I κ B β affects TNF α synthesis we examined each step of TNF α production. Secreted TNF α was detectable by ELISA after 2 hours of LPS stimulation and by 4 hours was significantly impaired in *I κ B β ^{-/-}* TEPM (Fig. 2a). IL-6 production was equivalent (Fig. 2a). We examined the level of pro-TNF α by intracellular FACS and found there was very little pro-TNF α detected in the *I κ B β ^{-/-}* TEPMs even after 8 hours of LPS stimulation (Fig. 2b). The average amount of pro-TNF α produced was 2–3 fold higher in WT compared to *I κ B β ^{-/-}* TEPM (Fig. 2c). Consistent with this difference in protein levels, steady-state TNF α was decreased 2–6 fold in the *I κ B β ^{-/-}* TEPM compared to WT cells (Fig. 2d). Although TNF α mRNA is known to be regulated^{22,23}, there was no difference in TNF α mRNA stability between WT and *I κ B β ^{-/-}* TEPM (Supplementary Fig. 5b). Therefore, I κ B β promotes TNF α transcription.

To understand how I κ B β affects TNF α transcription, we investigated which NF- κ B subunits were associated with I κ B β in macrophages. It is known that I κ B β associates with p65:p50 and c-Rel:p50 complexes²⁴ through direct binding to p65 and c-Rel but not p50⁶. However, we found that I κ B β could be immunoprecipitated only with p65 and c-Rel, but not p50 (Fig. 3a). Both immunoprecipitations with anti-p65 and anti-c-Rel antibodies pull down I κ B β , I κ B α and p50. Thus, there are p65:p50 and inducible c-Rel:p50 complexes that are associated with I κ B α or other I κ Bs, but not I κ B β . Reciprocal immunoprecipitation of p65 with c-Rel and both p65 and c-Rel with I κ B β suggests a p65:c-Rel heterodimer associated with I κ B β (Fig. 3b). To demonstrate the association of I κ B β with p65:c-Rel, we performed sequential immunoprecipitations by first immunoprecipitating I κ B β and then immunoprecipitating the eluted I κ B β complexes with anti-c-Rel antibody. The presence of p65 in the anti-c-Rel immunoprecipitate confirms the presence of I κ B β :p65:c-Rel complex (Fig. 3c). The I κ B β :p65:c-Rel complex was found in nuclear extracts suggesting that this could be a transcriptionally active complex. We had previously reported¹⁰ that I κ B β exists in two phosphorylation states: a hyperphosphorylated state in quiescent, unstimulated cells, and a hypophosphorylated newly synthesized state in LPS stimulated cells (Fig. 3c and Supplementary Fig. 5a). In the co-immunoprecipitation experiments shown here we found that both forms of I κ B β can bind p65 and c-Rel, although the hypophosphorylated form predominates in the I κ B β :p65:cRel complex following LPS stimulation.

There are four κ B sites upstream of TNF α coding region, three of which are crucial for NF- κ B dependent TNF α expression²⁵. Therefore, we performed chromatin immunoprecipitation (ChIP) with anti-p65, anti-c-Rel and anti-I κ B β antibodies in RAW264.7 cells and monitored the region encompassing these three κ B sites. Following LPS stimulation, TNF α promoter region DNA is enriched by p65, c-Rel and I κ B β antibodies by 56, 70 and 7 fold respectively (Fig. 3d). In contrast, I κ B β is not recruited to the IL-6 promoter following LPS stimulation while p65 and c-Rel are recruited as expected (Fig 3d). Recruitment of p65, c-Rel and I κ B β to the TNF α promoter was also confirmed in WT bone marrow derived macrophages (BMDM; Fig 3e). In the *I κ B β ^{-/-}* BMDM, both p65 and c-Rel are recruited normally to the TNF α promoter. However, when we performed immunoprecipitation with anti-p65, c-Rel and I κ B β are pulled down in WT but not *I κ B β ^{-/-}* BMDM (Fig. 3f). Therefore, p65 and c-Rel fail to form a stable complex in *I κ B β ^{-/-}* cells. Thus, the p65 and c-Rel recruited to the TNF α promoter in *I κ B β ^{-/-}* cells is not a p65:c-Rel complex. These data suggest that optimal

TNF α transcription requires a ternary complex of I κ B β :p65:c-Rel binding to the TNF α promoter.

In order to identify the κ B site for p65:c-Rel binding we performed EMSAs using the three κ B sites from the TNF α promoter as probes (κ B2, κ B2a and κ B3, Supplementary Fig. 5b). We identified two distinct gel-shift patterns. κ B3 and κ B2a show two major bands (only κ B3 is shown in Fig. 3g) while κ B2 shows three major inducible shift bands. The components of the bands were identified by super-shift assay (Fig. 3g, right panel). The top band in the κ B2 gel-shift is mostly p65:c-Rel. Interestingly, the κ B2 site possesses features predicted to favor p65:c-Rel binding (Supplementary Fig. 5c). Similar κ B binding sites in the CD40 and CXCL1 promoters also demonstrated coordinate recruitment of I κ B β , p65, and c-Rel (Supplementary Fig. 5d). Furthermore, deletion of the κ B2 site from a TNF α promoter reporter abrogated I κ B β -dependent reporter gene expression (Supplementary Fig. 6). In *I κ B β ^{-/-}* BMDM, the p65:c-Rel complex binding to the κ B2 in EMSA assays is missing (Fig. 3h), in agreement with the immunoprecipitation result. Therefore optimal TNF α transcription requires a p65:c-Rel complex, stabilized by hypophosphorylated I κ B β , binding to the κ B2 site in the TNF α promoter.

To identify other genes affected by I κ B β deficiency, we examined gene expression profiles in WT and *I κ B β ^{-/-}* BMDM. As expected, TNF α and I κ B β are among the genes whose expression is affected by I κ B β deficiency while IL-6 and IL-1 β are not affected (Fig. 4a). Of the genes whose expression is reduced in the *I κ B β ^{-/-}* cells we identified 14 with expression patterns resembling TNF α (Fig. 4b). The expression of these genes was also reduced in p65, c-Rel or p65/c-Rel knock-out fetal liver macrophages suggesting that LPS-induced expression of these genes might depend on a mechanism similar to TNF α (data not shown). The expression of TNF α , IL-1 α , IL-6 and IL-1 β in response to LPS was further examined by RNase protection (Fig. 4c) and qRT-PCR assays (Supplementary Fig. 7) demonstrating that the reduction in persistent expression of TNF α in *I κ B β ^{-/-}* cells is unique. Reduced *IL12b* mRNA and protein secretion in the knockout TEPM was confirmed by qRT-PCR (Fig. 4d) and ELISA (Fig. 4e). Notably, transcription of *IL12b*, which has a κ B site similar to κ B2 of TNF α (Supplementary Fig. 5c), has previously been shown to require c-Rel and be partially dependent on p65²⁶. Thus, only a select group of NF- κ B dependent genes are diminished similarly to TNF α upon I κ B β deletion. As TNF α plays a key role in inflammation, we wanted to test whether *I κ B β ^{-/-}* deletion would affect the course of inflammatory diseases.

Rheumatoid arthritis (RA) is a common inflammatory disease with morbidity resulting from ongoing release of pro-inflammatory cytokines, including TNF α , and consequent destruction of joint tissue²⁷. Previous studies have shown that NF- κ B plays a key role in mouse models of arthritis and blocking NF- κ B has a dramatic effect in preventing disease^{28,29}. RA can also be effectively treated by anti-TNF α therapies, although there are significant side-effects³⁰. The ability to block only persistent TNF α expression would be therapeutic without blocking beneficial TNF α responses including the expression of innate immune response genes. We therefore tested whether the lack of I κ B β altered the course of collagen-induced arthritis (CIA), a well-characterized mouse model of RA.

To induce CIA we immunized DBA/1J mice with bovine type II collagen. *IκBβ*^{-/-} mice displayed delayed onset, lower incidence and decreased severity of CIA (Fig. 4f and Supplementary Fig. 8). Inflammation in the WT mice extended from the paws and digits to the ankle joints and distally through the limb (data not shown). In contrast, *IκBβ*^{-/-} mice showed minimal visual signs of paw and joint swelling (Supplementary Fig. 8). Serum TNFα was markedly decreased in *IκBβ*^{-/-} mice while other pro-inflammatory cytokines were not significantly affected (Fig. 4g and Supplementary Fig. 9). Therefore the absence of IκBβ limits the progression and severity of arthritis by reducing the chronic production of TNFα.

The results presented above demonstrate a dual role for IκBβ: during the early stages of LPS stimulation, NF-κB complexes released by IκBβ degradation contribute to the initial expression of TNFα (Supplementary Fig. 1). Then, newly synthesized hypophosphorylated IκBβ facilitates the formation of IκBβ:p65:c-Rel complexes which selectively bind to the κB2 site in the TNFα promoter augmenting transcription. As shown in the gene chip and RNase protection assays, this is a relatively selective function and *IκBβ*^{-/-} mice are, therefore, otherwise normal. Hence targeting IκBβ might be a promising new strategy to treat chronic inflammatory diseases such as arthritis.

Methods summary

Mice

IκBβ deficient mice were generated by standard homologous recombination in the CJ7 ES cell line using a targeting construct that replaced exon 2 through exon 5 with a G418-resistance gene. Screened ES cell clones were injected into blastocysts derived from C57BL/6 mice gave rise to *IκBβ*^{-/+}/*IκBβ*^{+/+} chimeras. Germline transmission of the disrupted allele was obtained and verified by Southern blotting and PCR, and mice were backcrossed at least 10 generations onto the B57BL/6 background. Mice were backcrossed at least 8 generations onto the DBA background for CIA experiments. Mice were maintained in pathogen-free animal facilities at Yale Medical School.

Cells

WT and *IκBβ* knockout MEFs were generated from E12.5 embryos following timed breeding of *IκBβ*^{+/-} animals. TEMP₃ were obtained from 6- to 8-week-old littermate mice three days after intraperitoneal injection with thioglycollate. BMDM were harvested by standard protocols and differentiated with 30% L929 supernatant-conditioned media.

Biochemistry

Cell fractionation, western blotting, EMSA, and immunoprecipitations were performed as previously described unless otherwise indicated⁶.

LPS-induced shock

LPS-induced shock was tested by intraperitoneal injection of 50 ug/g body weight LPS and monitoring for survival. In a separate identical experiment, the mice were bled at 1 hr and 2

hr after LPS treatment and the concentration of TNF- α , IL-6 and IL-1 β in the serum was measured by ELISA.

Intracellular cytokine analysis

Pro-TNF α levels were analyzed in LPS stimulated TEMPs cells following LPS stimulation and brefeldin-A treatment. TNF α was detected following cell permeabilization using standard intracellular cytokine staining and flow cytometry.

qRT-PCR

RNA expression was quantified by quantitative two-step SYBR real-time RT-PCR, and relative mRNA levels were obtained by normalizing the readout for each specific gene by that of β -actin.

Microarray Analysis

Microarrays for gene expression analyses were performed on BMDMs stimulated with LPS and Affymetrix Mouse genome 430A 2.0 arrays as per the manufacturers protocol.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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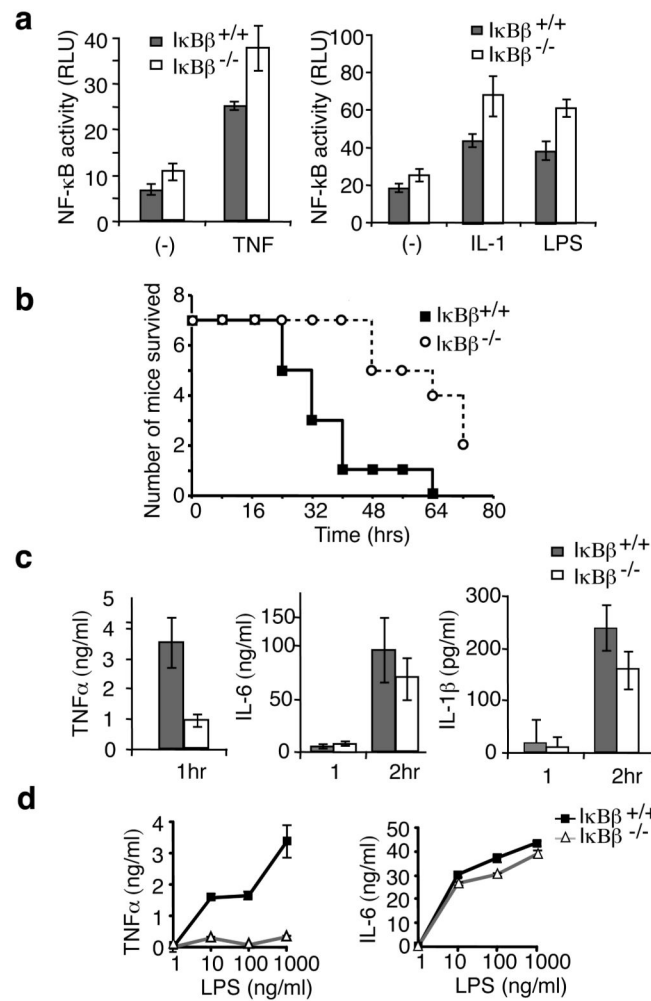


Figure 1. Mice lacking $I\kappa B\beta$ are resistant to LPS-induced endotoxin shock

a, WT and $I\kappa B\beta^{-/-}$ MEF cells transfected with pBIIx-luc reporter and Renilla luciferase vectors were treated with TNFα, IL-1β or LPS for 4 hours and analyzed for luciferase activity. Results are expressed as relative luciferase unit (RLU) normalized by Renilla luciferase activity; error bars indicate \pm s.d (n=3). **b**, Age and sex matched mice received intra-peritoneal injection of LPS and survival rates were scored every 8 hours for 3 days (n=7). **c**, Serum TNFα, IL-6 and IL-1β 1 hour and/or 2 hour after IP injection of LPS was examined by ELISA; error bars indicate \pm s.d (n=5). **d**, TEPMs from littermate mice were treated for 20 hours with LPS as indicated, and TNFα and IL-6 in the media was determined by ELISA; error bars indicate \pm s.d (n=3).

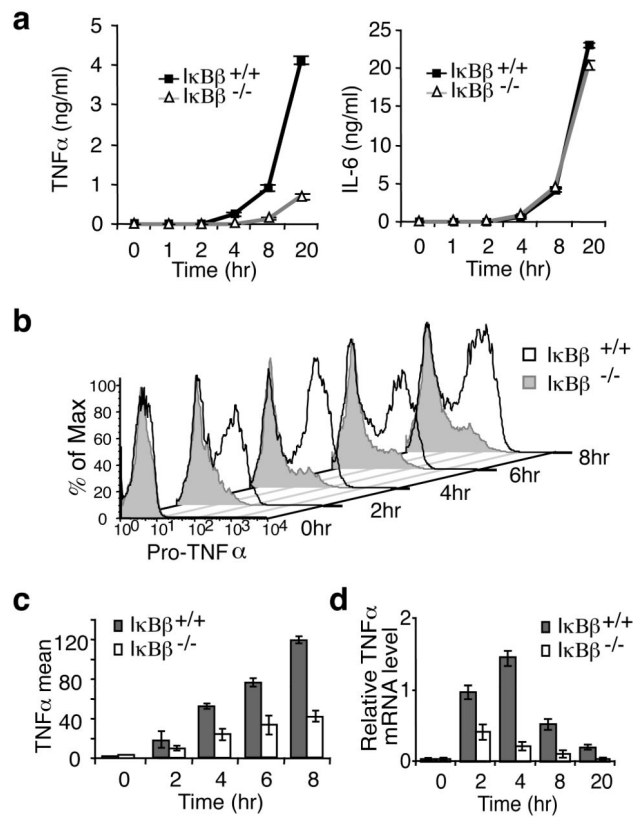


Figure 2. Deficient TNF α transcription in $I\kappa B\beta^{-/-}$ macrophages

a, TEPMs from littermate WT and $I\kappa B\beta^{-/-}$ mice were treated with LPS and secreted TNF α and IL-6 were determined by ELISA; error bars indicate \pm s.d. (n=3). **b**, TEPMs from littermate mice were treated as in (a) in the presence of Brefeldin A, and intracellular pro-TNF α was examined with flow cytometry. **c**, Intracellular pro-TNF α production was examined as in B with macrophages isolated from 3 pairs of littermate mice; error bars indicate \pm s.d. **d**, TEPMs were stimulated with LPS as in A and relative TNF α mRNA level was determined by qRT-PCR; error bars indicate \pm s.d (n=3).

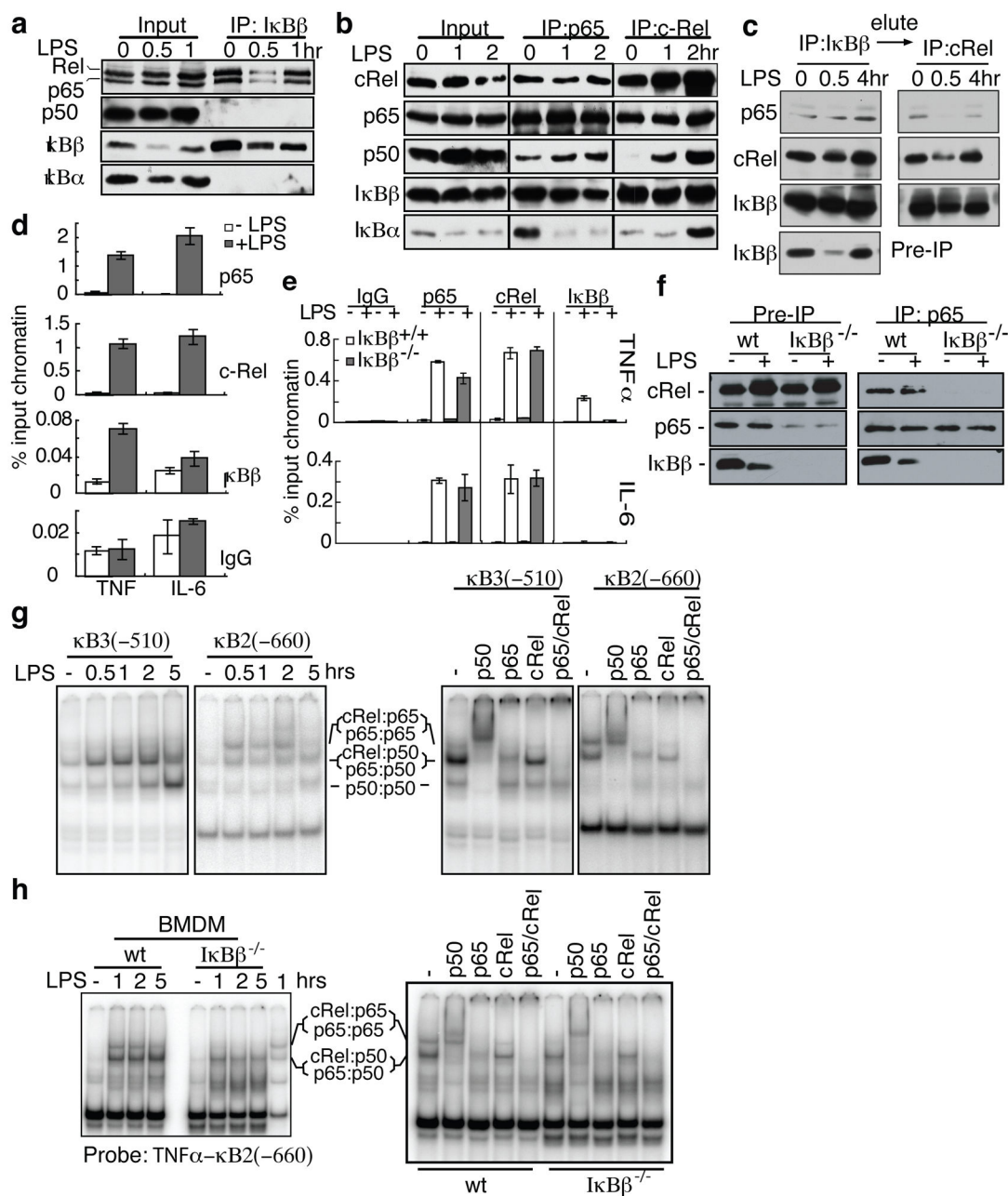


Figure 3. IκBβ is recruited to the promoter of TNFα together with P65 and c-Rel

a,b, Raw264.7 were stimulated with LPS and immunoprecipitated (IP) with anti-IκBβ (a), anti-p65 (b) or anti-c-Rel (b) antibodies and immunoblotted (IB) as indicated. **c**, LPS-stimulated Raw264.7 lysates were immunoprecipitated with anti-IκBβ; eluted with IκBβ peptide; immunoprecipitated with anti-c-Rel antibody; and immunoblotted as indicated. **d**, Raw264.7 lysates were subjected to ChIP as indicated and analyzed by qPCR targeting TNFα and IL-6 promoter κB sites; error bars indicate \pm s.d (n=3). **e**, ChIP was performed as in (d) on WT and IκBβ^{-/-} BMDM treated with LPS for 2 hours; error bars indicate \pm s.d (n=3). **f**, BMDM treated as in (e) were immunoprecipitated with anti-p65 antibody. **g**, RAW264.7 were treated with LPS and nuclear extracts were subjected to EMSA TNFα κB3

or κ B2 probes. Super shifts were performed using cells stimulated for 1hr. **h**, BMDM were treated with LPS and EMSA and supershifts with the κ B2 probe were performed as in (g).

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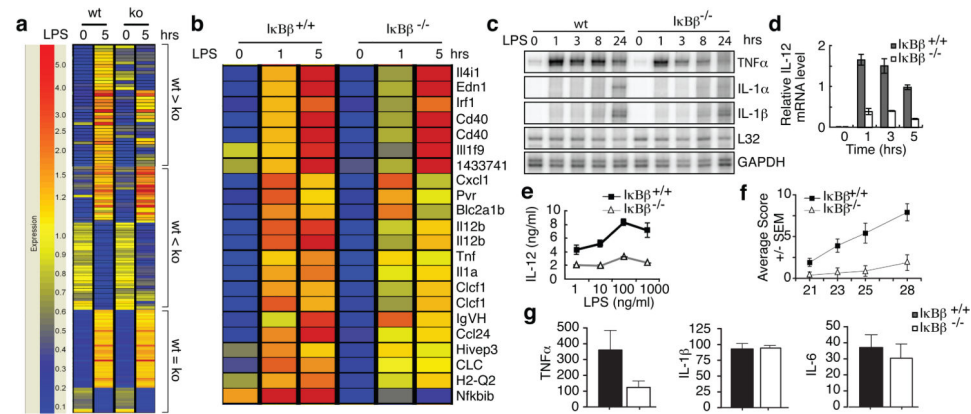


Figure 4. $\text{I}\kappa\text{B}\beta$ knockout selectively affects only certain LPS responsive genes and attenuates collagen induced arthritis

a, LPS responsive genes whose expression is either down-regulated, up-regulated or unchanged in $\text{I}\kappa\text{B}\beta^{-/-}$ BMDM. **b**, Host-pathogen interaction genes that are $\text{I}\kappa\text{B}\beta$ dependent and LPS responsive genes whose expression pattern resembles $\text{TNF}\alpha$. **c**, RNase protection assay using WT and $\text{I}\kappa\text{B}\beta^{-/-}$ BMDM stimulated with LPS. **d**, IL-12b relative mRNA level determined by qRT-PCR in samples prepared as in (c); error bars indicate \pm s.d. (n=3). **e**, ELISA for IL-12p40 secreted from WT and $\text{I}\kappa\text{B}\beta^{-/-}$ TEMP stimulated with LPS for 20 hours; error bars indicate \pm s.d. **f**, Arthritis clinical scoring in WT (n=10) or $\text{I}\kappa\text{B}\beta^{-/-}$ (n=8) DBA mice; error bars indicate \pm SEM. **g**, Serum $\text{TNF}\alpha$, IL-1 β , and IL-6 levels in WT or $\text{I}\kappa\text{B}\beta^{-/-}$ DBA mice in (f); error bars indicate \pm SEM.